IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Garth J.S. Cooper	Art Unit:	To be assigned
Serial No.:	715,031	Examiner:	To be assigned
Filed :	June 10, 1991		
For :	TREATMENT OF DIABETES MELLITUS		

DECLARATION UNDER 37 C.F.R. §1.132

I, Andrew Arthur Young, declare that:

1. I am Principal Scientist and Director, Department of Physiology, at Amylin Corporation, 9373 Towne Centre Drive, San Diego, California 92121.

2. As described in my Curriculum Vitae, which is attached as Exhibit A, I have received the following degrees from the University of Auckland: Bachelor of Science in Human Biology in 1974; Master of Science with Honours (First Class) in Physiology in 1978; Bachelor of Medicine and Bachelor of Surgery (equivalent to United States M.D.) in 1979; and, Doctor of Philosophy in Physiology in 1985. I taught physiology at the University of Auckland for six years from 1979-1989 and my research has included investigations into the mechanisms of insulin resistance. Since joining Amylin Corporation in 1989, I have been further investigating the physiologic roles of amylin in various standard animal models used in diabetes research.

TREATMENT OF HYPOGLYCEMIA WITH AMYLIN

3. In conjunction with my research, I conducted and supervised a series of experiments in order to evaluate the use of amylin for the treatment of hypoglycemia, such as that induced by insulin therapy. The experiments were conducted as follows:

There were 14 male Harlan Sprague Dawley rats in 3 treatment groups. Animals were housed at 22.7±0.86°C in a 12:12 hour light:dark cycle (experiments being performed during the light cycle) and fed and watered ad libitum (Diet LM-485, Teklad, Madison, WI).

Two hours after surgery and instrumentation, the rats were infused with an intravenous bolus of 100mU of insulin (Humulin-R; Eli Lilly, Indianapolis, IN) followed by an intravenous infusion of 50mU/hr for the next 6 hours. Two hours after starting insulin, they were given a bolus intravenous injection of saline, 100μg rat-amylin or 100μg glucagon as detailed:

1. **Saline Control:** n=5, age 80±5d, mass 318±10g, fasted 20.9±0.5hr. Administered 0.1mL normal saline.

2. **Amylin:** n=5, age 79±5d, mass 322±9g, fasted 20.9±0.5hr. Administered 100μg r-amylin (Bachem, Torrance, CA) in 0.1mL saline.

3. **Glucagon:** n=4, age 87±1d, mass 326±8g, fasted 19.9±0.7hr. Administered 100μg glucagon for injection (Eli Lilly, Indianapolis, IN) in 0.1mL of supplied diluent.

Anaesthesia was induced in 18-hr fasted rats using 5% halothane which was then maintained at 2% during surgery and at 0.8-1% during subsequent metabolic recordings. Tracheotomy and cannulation of right femoral artery and vein were performed and core temperature controlled with a thermoregulator (Model 73A, YSI, Yellow Springs, OH) which switched a heated operating table.

To monitor arterial pressure the femoral arterial line was connected to a pressure transducer (Spectramed P23XL transducer, Model 13-4615-58 amplifier, Gould, Cleveland, OH) and perfused with heparinized saline (2U/ml) at 3.0 ml/hr. The femoral venous line was used for acute (bolus) injections.

Arterial samples were drawn every 30 minutes from -0.5

until 4 hours after the insulin bolus injection, and also at 5 and 6 hours. Samples were collected into heparinized capillaries and separated plasma analyzed for glucose using immobilized enzyme chemistry (glucose oxidase, Analyzer model 2300-STAT, YSI, Yellow Springs, OH).

4. The results of the experiments are presented in a graph which is attached as Exhibit B. It shows that in animals made hypoglycemic as described above with insulin, amylin is an effective hyperglycemic agent, that is, amylin can be introduced in order to treat hypoglycemia and aid in normalizing blood glucose levels.

5. Exhibit B also shows that amylin was more effective in comparison with glucagon in these experiments. Glucagon is a single-chain polypeptide hormone containing 29 amino acid residues which also causes an increase in blood glucose concentration and has long been used in the treatment of hypoglycemia. Glucagon is known to act only on liver glycogen, converting it to glucose and thereby counteracting hypoglycemia. It is believed that the effect of amylin in overcoming the insulin-induced hypoglycemia in these experiments was greater than that of glucagon at least partly because overnight fasted animals were used in the experiments and those animals can be expected to have depleted liver glycogen. Amylin also causes increases in blood glucose levels in fed test animals (data not shown), although not as large as those in fasted animals. Increases in blood glucose levels in response to glucagon are higher in fed animals than in fasted animals.

6. These experiments demonstrate the operativeness of amylin for use in the treatment of hypoglycemia in standard test animals, and will be recognized by those in the field as being predictive of utility in the treatment of hypoglycemia in humans.

TREATMENT OF DIABETES MELLITUS WITH AMYLIN

7. I also conducted and supervised a series of experiments in which a standard animal model for diabetes, streptozotocin-diabetic rats, were treated with daily injections of amylin as well as insulin. The procedures used were as follows:

There were 68 male Harlan Sprague Dawley rats (mass 301±3g) in 8 treatment groups. Animals were housed at 22.7±0.8°C in a 12:12 hour light:dark cycle (experiments being performed during the light cycle) and fed and watered ad libitum (Diet LM-485, Teklad, Madison, WI). The rats were sacrificed for harvesting of livers 4 to 5 hours into the light cycle. The treatment groups were as follows:

Group 1: Streptozotocin-diabetic animals, insulin-only treatment (n=11). Animals were injected with streptozotocin (Sigma Chemical Company, ST. Louis MO: Sigma S0130) dissolved in water in a dose of 65mg/kg into the lateral tail vein. Upon exhibiting 5% glycosuria (Chemstrip uGK, Boehringer-Mannheim, FRG), rats were commenced upon a sliding-scale daily insulin treatment regime (Humulin-Ultralente, Eli Lilly, Indianapolis, IN) aimed towards maintaining aketonuria (by Chemstrip) but 5% glycosuria in order to optimize survival. Following one week of established diabetes, animals received once daily subcutaneous injections of amylin vehicle (water for injection) for 5 days given at the time of the insulin injection.

Groups 2-6: Streptozotocin-diabetic animals, insulin+amylin treatment. These animals were treated identically to those in group 1 except that the daily subcutaneous injection contained 3μg (n=5); 10μg (n=12); 30μg (n=5); 100μg (n=5) of rat amylin (Bachem lot #WG485). The bioactivity of the peptide used in these experiments was first verified by bioassay, using inhibition of

insulin-stimulated radioglucose incorporation into glycogen in the isolated stripped rat soleus muscle. The EC50 derived for the peptide used was 6.2nM (±0.2 log unit). The insulin dose in each of the groups of diabetic animals averaged 1.67U/animal/day. There were no observable differences in the required management of the different groups.

Group 7: Normal animals (n=10). These animals were derived from the same stock and housed under the same conditions for the same time as those in groups 1-6, but were given no injections.

Group 8: Streptozotocin-diabetic animals, no treatment (n=10). These animals were made diabetic as were groups 1-6, but received neither insulin nor amylin.

8. Liver glycogen content was measured in rats having free access to food up to the time of sacrifice. Glycogen content for the 8 groups of animals are shown in the attached Exhibit C. Diabetic animals on no therapy showed a 67% decrease in liver glycogen concentration compared to normal rats (2.86 vs 8.6 mg/g, $P<0.001$). Diabetic animals receiving insulin replacement had a 35% decrease in liver glycogen compared to normal rats (5.6 vs 8.6 mg/g, $P<0.01$). In insulin-treated diabetic rats supplemented with daily amylin, there was a dose-dependent increase in liver glycogen concentration to normal, i.e., above that in rats treated with insulin alone ($P<0.05$, 10μg/day; $P<0.02$, 30μg/day; $P<0.01$, 100μg/day). The liver glycogen concentration in animals receiving 10, 30 and 100μg amylin per day was not significantly different from that in normal animals ($P>0.3$), but was significantly greater than insulin-treated animals receiving no amylin.

9. It is known that insulin-dependent diabetic rats have a marked depletion of liver glycogen that is only partly corrected

with insulin. In accord with that observation, these experiments show that in streptozotocin-diabetic rats treated with insulin replacement, liver glycogen was 35% depleted. However, five consecutive once daily subcutaneous injections with amylin dose-dependently restored liver glycogen to normal levels. In other words, combined replacement of amylin and insulin can restore normal levels of liver glycogen in this animal model of diabetes. Such full restoration is not achieved by insulin alone.

10. These experiments demonstrate the operativeness of amylin for use in the treatment of diabetic animals, and will be recognized by those in the field as being predictive of utility in conjunction with insulin therapy in humans, including insulin therapy in the treatment of diabetes mellitus. Diabetic subjects will benefit from enhanced liver glycogen stores that can be mobilized in the form of glucose to prevent blood sugar levels from becoming too low.

11. These experiments also demonstrate the operativeness of agonists of amylin, such as CGRP and other compositions having the properties of amylin, for use in the treatment of hypoglycemic and diabetic animals, and will be recognized by those in the field as being predictive of utility in the treatment of hypoglycemia in humans, and in conjunction with insulin therapy in humans, including insulin therapy in the treatment of diabetes mellitus.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the above-identified U.S. patent application or any patent issued thereon.

Signature: Andrew A. Young Date July 22, 1991

Andrew A. Young

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